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(RESEARCH ARTICLE)



## Comparative assessments of polymerase chain reaction (PCR) assay of repetitive sequence (RLEP) and proline rich antigen (PRA) gene targets for detection of *Mycobacterium leprae* DNA from paucibacillary (PB) and multibacillary (MB) patients in Côte d'Ivoire (CI)

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### Abstract

Several *Mycobacterium leprae* gene targets could be used for leprosy diagnostic. PCR sensitivity of some gene targets for specific clinical isolates has not yet been established in Côte d'Ivoire. The present study was conducted to compare the sensitivity of the *Pra* gene and *RLEP* targets in the detection of *M. leprae* from nasal mucus and ear dermal pulp fluid samples from paucibacillary and multibacillary patients. Leprosy patients were classified into Paucibacillary and Multibacillary types. DNA extraction from samples was performed by a guanidine thiocyanate method. PCR technique was performed for *M. leprae* detection from samples. 84.94 % were positive for *RLEP* target while 69.94 % were positive for the *Pra* gene target. For paucibacillary cases with bacteriological index negative, 180 one 258 (69.76 %) were positive for the *RLEP* target and 193 one 258 (74.80 %) were positive for *Pra* gene target. Concerning multibacillary cases with bacteriological index positive samples, 100% of the samples were positive for *RLEP* target and 65.21 % for *Pra* gene target. The *Pra* gene target showed a large difference in detection of *M. leprae* between paucibacillary and multibacillary forms in leprosy patients. In conclusion, PCR-*Pra* could be a useful tool for diagnosis of leprosy, mainly in cases of Paucibacillary leprosy or when bacilloscopy is negative. The PCR-*RLEP* appear to be the best target for early diagnosis of leprosy.

Keywords: Leprosy; *Mycobacterium leprae*; molecular diagnostic; *Pra* gene; *RLEP*; Paucibacillary; Multibacillary

### 1. Introduction

Leprosy is a chronic infection disease of skin and the peripheral nerves that can entail the disabilities and a social stigmatization [1]. This disease is caused by an environmental mycobacterium, *M. leprae*, belonging to the same family that bacteria responsible of the tuberculosis and Buruli ulcer. Leprosy is a neglected tropical disease like Buruli ulcer and yaws which are also skin diseases. The diagnosis of leprosy is challenging due to its complex immune response that leads to a variety of clinical forms. So far, *M. leprae* cannot grow in vitro, and this is a limiting factor to study the disease [2]. The diagnosis is based on clinical evaluation of patients, whereas histopathological analysis and bacilloscopy are complementary diagnostic exams. Because of a long period of incubation of the bacterium on the one hand and following the fact that some lesions are often different to usual clinical aspects of the leprosy, it became difficult to diagnose the disease to early stage. So Molecular tools of diagnosis [3] permitting to confirm the disease have been developed so that the patients can benefit quickly from an appropriate therapy and avoid handicaps thus and interrupt transmission of

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the germ in the population. During the last 30 years, several PCR methods have been developed to amplify different targets of *M. leprae* genome that from different type of clinical sample [4]. Among the gold standard targets we could cite repeated elements *RLEP* and the encoding rich proline antigen *Pra* gene. *RLEP* is a sequence repeated in the genome of *M. leprae* was revealed very sensitive and specific to *M. leprae* and detect weak quantity of DNA purified of *M. leprae* even [5]. Also The use of *RLEP* has the advantage of being more sensitive than targets located in other gene regions because it provides multiple copies [4]. The *Pra* target is a gene kept in the bacterium and coding for a protein rich in Proline. It has been used for the identification of *M. leprae* from urine and nasal secretions in patients affected by the disease in a recent survey [6]. The authors showed that the *Pra* gene would constitute a better target for the diagnosis of *M. leprae* in samples with weak bacterial loads. Côte d'Ivoire is a country located in West Africa. Like some countries in the world, eliminating leprosy is a public health problem despite the availability of Multi Drug Therapy (MDT). Indeed, about 1000 new cases are detected every year and the rate of infirmity of degree two rises to 20 % [7], so the disease is far from being eradicated. In CI, the diagnosis of the disease is essentially clinical, microbiological approaches being restricted to the bacilloscopy. The eradication of a disease being also conditioned by the performance of the diagnosis, we evaluated the *RLEP* and *Pra* targets in the molecular detection of *M. leprae* in paucibacillary and multibacillary patients and from the nasal secretions and ear dermal pulp fluid.

## 2. Material and methods

### 2.1. Study population

One hundred fifty five (155) patients clinically diagnosed with leprosy were identified at Raoul Follereau Institute of Côte d'Ivoire. Leprosy patients were classified by physicians using clinical signs and bacteriological criteria and assigned to the following leprosy forms according to WHO classification: paucibacillary (PB, N= 86) and multibacillary (MB, N= 69). The variables age and gender were analyzed.

#### 2.1.1. Ethics statement

This study was approved by the National Ethic committee for research of Côte d'Ivoire under the approval number N/Réf: N°140/MSHP/CNER-km. All participants approved the research protocol and signed the informed consent after read of the study information notice.

#### 2.1.2. Biological sample

465 samples consisted of nasal secretions (155) and ear dermal pulp fluids (310) were collected from 155 patients clinically confirmed for leprosy. Ziehl–Neelsen (ZN) staining method for 155 nasal secretions, 310 exudates of ear lobules samples and Bacteriological Index (BI) was calculated for all patients. A part of remaining of samples were stored in sterile microtubes containing 50 mL of sterile PBS 1 X (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>) and preserved at -20 °C until use.

### 2.2. DNA extraction from nasal secretions and ear dermal pulp fluids samples

DNA was extracted from samples by a guanidine thiocyanate modified method as described by Chomczynski *and al.* [8]. Briefly 300 µL of each sample (previously pretreated with PBS buffer) was treated with 800 µL of lysis buffer (5 M guanidine thiocyanate, 50 mM Tris, pH = 8.0, 10 mM EDTA, 5 % 2 Mercaptoethanol, 2 % Triton X-100). Subsequently, mixture was centrifuged at 10,000 rpm for 3 min at 4 °C. Supernatant was then transferred into a sterile Eppendorf tube to which 400 µL of isopropanol were added. Been added, mixture was centrifuged at 10,000 rpm for 5 min at 4 °C. The pellet was resuspended in 300 µL of buffer (10 mM Tris, pH = 8.0, 1 mM EDTA) to which 110 µL of sodium acetate (3M) and 800 µL of absolute 100% non-icing alcohol were added. Mixture was incubated at 4 °C for 30 min and then centrifuged at 10,000 rpm for 5 min at 4 °C. This was dried at 50 °C for 30 min. It was then resuspended with 150 µL of elution buffer and incubated at 55 °C for 5 min. Suspension was cooled to room temperature for 5 minutes before being stored at -20 °C.

### 2.3. Polymerase Chain Reaction (PCR)

DNA extracted from *Mycobacterium ulcerans* was used as an internal control for the specificity of the *RLEP* and *Pra* gene PCR.

#### 2.3.1. PCR for Detecting *Pra* gene

*M. leprae* specific *Pra* gene primers were selected as described by Kamalanathan *and al.* [6] It's a nucleotide sequence of a 151-bp proline-rich region fragment gene that encodes the 36-kDa antigen of *M. leprae* (*GenBank accession No.*

X65546.1). The PCR-*Pra* were carried out using 5  $\mu$ L of DNA template in 25  $\mu$ L of a reaction mixture containing 10 mM of dNTPs (Invitrogen), Gotaq G2 Flexi DNA polymerase (Promega, Madison, WI USA) of 5U /  $\mu$ L, 10  $\mu$ M of each primer and 5X GoTaq buffer (Promega, Madison, WI USA). The primers used for amplification of the *Pra* gene were Pra1: 5'-ATCCGCTCACTGCCTAAGGA-3' and Pra2: 5' TCGGTGATATCAGTGACGGAC- 3'. Amplification was performed in a Perkin Elmer 9700 thermal cycler (Applied Biosystems) according to the following program: An initial denaturation at 95 °C for 5 min, following by 35 cycles of denaturation at 95 °C for 60 s, annealing at 58 °C for 60 s, an extension stage at 72 °C for 60 s, and a final elongation phase at 72 °C for 10 min.

### 2.3.2. PCR for detecting Repetitive Element RLEP

PCR *RLEP* was realized in a final reaction mixture of 25  $\mu$ L consisted of 5 U /  $\mu$ L of Gotaq G2 Flexi DNA polymerase (Promega, Madison, WI USA), 5X GoTaq Buffer (Promega), 10 mM of each dNTPs, 0.25 mM MgCl<sub>2</sub>, 10  $\mu$ M concentrations of the primers and 5 $\mu$ l of DNA template. Primers REP 7 (5'- TGAAGGCGATATCGATGCAG - 3') and REP 8 (5' ATCCCTTGACCATTTCTGC - 3') were used to amplify *M. leprae* repetitive elements *RLEP* gene (545 bp) according to the program as following: 40 cycles consisted of a denaturation 30 s at 94 °C, annealing: 1 min at 57 °C and elongation: 1 min at 72 °C for.

### 2.3.3. PCR products revelation and analysis

PCR products were revealed with a Geldoc EZ imager (Bio-Rad) after an electrophoresis in 2% agarose gel (Promega, Madison, USA) stained with Syber safe (0.5  $\mu$ g/mL). A 100 bp DNA Ladder (Promega, Madison, USA) was used as a molecular marker. Samples were considered positive for *M. leprae* when a single PCR product of 545 bp and 151 pb was observed in an amplified product by the PCR-*RLEP* or PCR-*Pra*, respectively. When the PCR-product showed to be negative, the amplification was repeated using a DNA template diluted to 1:10. Patients samples were considered negative when no amplification was observed and that amplification of positive and negative controls were positive and negative respectively. Result datas were analyzed using the XLstat test (software BioStat 5.0) and were considered significant for  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Results

#### 3.1.1. Clinical, bacilloscopy and demographic characteristics of leprosy patients

**Table 1** Demographic characteristics of leprosy patients according to the leprosy form and the bascilloscopy results

N°	Characteristics	Types	Patients (n=155)	
			No	%
1	Gender	Male	81	52.26
		Female	74	47.74
2	Age	0-12	5	3.23
		13-20	17	10.97
		21-65	127	81.94
		> 65	6	3.87
3	WHO Classification	PB	86	55.48
		MB	69	44.52
4	BI	0	96	61.93
		1	23	14.84
		2	19	12.26
		3	8	5.76
		4	6	3.87
		5	3	1.94

BI = Bacteriological Index, PB = Paucibacillary leprosy, MB= Multibacillary leprosy, WHO =World Health Organization

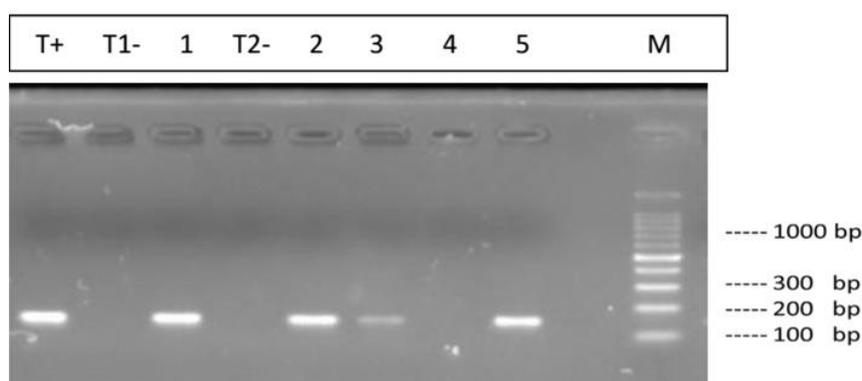
Out of the 155 patients included in this study, 47.74 % were females and 52.26 % were males. The average age of the patients with leprosy was 34,  $64 \pm 16.66$  years (range, 9-72 years). The patients were diagnosed with leprosy by clinical and bacilloscopy analysis (refer to table 1)

### 3.1.2. Molecular confirmation of *M. leprae*

Both PCR-*RLEP* and PCR-*Pra* showed 100 % specificity for *M. leprae*. No amplification was observed using DNA template from *M. ulcerans* as using negative control. The PCR- *RLEP* and PCR-*Pra* results were described in table 2. The 2 % agarose gel electrophoresis revealed an amplification of 545 bp for the repeat sequence *RLEP* (refer to figure 1) and 151 bp for the *Pra* gene (refer to figure 2). Comparative evaluation of PCR positivity among leprosy patients indicated that 325/465 (70 %) were positive for *Pra* compared to 395/465 (85 %) for *RLEP*. On 465 samples, 281 (60.47 %) samples were positive for *Pra* and *RLEP*, 55 (11.80 %) samples were negative for *Pra* and *RLEP*, 114 samples (24.53 %) were negative for *Pra* and positive for *RLEP*, and 15 (03.20 %) samples were positive for *Pra* and negative for *RLEP*.

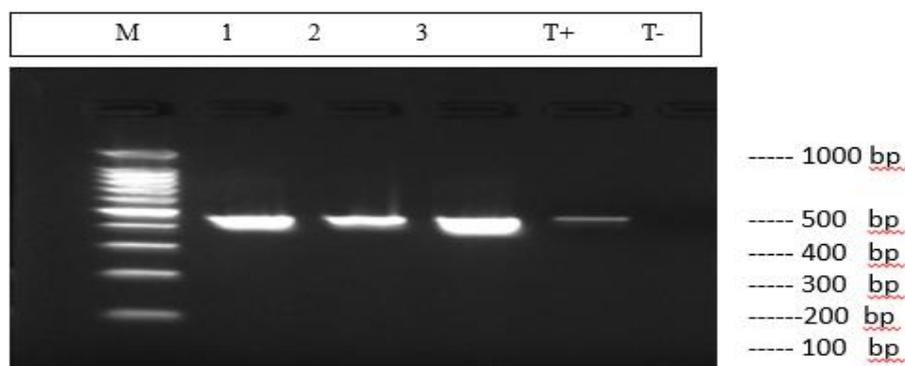
**Table 2** PCR positivity rates of the *RLEP* elements and *Pra* gene in leprosy patients

PCR target	PCR results		Total
	Positive(%)	Negative(%)	
<b>Pra gene</b>	325 (70 %)	140 (30 %)	465 (100 %)
<b>RLEP elements</b>	395 (85 %)	70 (15 %)	465 (100 %)



**Figure 1** PCR *Pra* agarose gel electrophoresis revelation

The *Pra* gene amplified region (151 bp) confirm the presence of *M. leprae* in samples. M: 100bp DNA Ladder, T +: Positive control, T1-: Negative control (*M. ulcerans* DNA), T2- : Negative control 2 (Nuclease free Water), 1: lep Adzop 18, 2: lep Lako 18, 3: lep Dalo 2018, 4: lep Zuen 18 and 5: lep Daou 18.



**Figure 2** PCR *RLEP* agarose gel electrophoresis revelation

The *RLEP* amplified elements (545 bp) confirm the presence of *M. leprae* in samples. M: 100bp DNA Ladder, T +: Positive control, T-: Negative control (*M. ulcerans* DNA), 1: lep Adzop 18, 2: lep Lako 18 and 3: lep Dalo 2018.

### 3.1.3. Comparison of PCR positivity for Pra and RLEP according to the clinical forms

The PCR-RLEP and PCR-Pra comparison according to the clinical forms of leprosy is described in table 3. Regarding the paucibacillary and multibacillary forms, 69.76 % (180/258) and 100 % (207/207) were *M. leprae* respectively positive for the RLEP target. The Pra target was able to detect 65.21 % (135/207) and 74.80 % (65/258) respectively for MB and PB forms.

**Table 3** RLEP and Pra PCR results according to the clinical leprosy form

Characteristic	PCR- RLEP		PCR- Pra	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Clinical form				
MB (n= 207)	207 (100 %)	0 (0 %)	135 (65.21 %)	72 (34.79 %)
PB (n= 258)	180 (69.76 %)	78 (30.23 %)	193 (74.80 %)	65 (25.19 %)

### 3.1.4. Comparison of Pra and RLEP PCR positivity according to the type of Sample

RLEP gene target was able to detect the presence of *M. leprae* in 75 % of nasal secretion samples and in 95 % of ear dermal pulp fluid samples. Pra target permitted to detect the signature of *M. leprae* in 100 % of nasal secretions ample and in 40 % of ear dermal pulp fluid samples (refer to table 4).

**Table 4** PCR results according to the PCR target and the type of sample

Sample type	Nasal secretion n= 155		Ear dermal pulp fluid n= 310	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
PCR targets				
RLEP	116 (74.83 %)	49 (25.16 %)	294 (94.83 %)	16(5.16 %)
PRA	155 (100 %)	0 (0 %)	124 (40 %)	186 (60 %)

## 4. Discussion

Despite the availability of an effective treatment, the transmission of leprosy continues. *M. leprae* the causative agent has a long incubation time and clinical signs are often difficult to recognize mainly in the early stage of the disease, or are assimilated to another skin disease. So the ignorance of the disease by population and the lack in the expertise in case recognition by clinicians are factors delaying the diagnosis [9]. Effectively diagnosis of leprosy has always been a difficult task for clinicians because disease follows an immunological spectrum in which paucibacillary forms are often misdiagnosed due to the absence of clear and obvious clinical manifestations [10]. So Polymerase chain reaction may be an important tool for the confirmation of leprosy suspected cases when clinical findings and slit skin smears are inconclusive [11]. PCR has proved to be an excellent support for diagnosis of leprosy as the works of Almeida *and al.* [12] have shown. The effectiveness of PCR in the detection of *M. leprae* DNA in various clinical samples like nasal swabs and other body fluids confirm our results. We detected *M. leprae* from nasal secretion and ear dermal pulp fluids with detection rates ranging from 74.83 % to 100 % and 94.83 % to 40 % respectively according to the targets used.

The variability of *M. leprae* genetic targets have been selected and analyzed for their sensitivity and specificity in a diagnostic context. So the genetic repeated elements RLEP and Proline rich Antigen gene Pra have been used in this study on two type of samples: nasal secretions and ear dermal pulp fluids. The RLEP target have demonstrated a higher sensitivity and specificity than other gene targets including the Pra gene. It was also reported previously that RLEP allowed also detection of low levels of *M. leprae* DNA [13]. Comparing the results on the basis of PCR detection rates we confirmed that RLEP target given better result than Pra target with respectively rate detection of 84.94 % versus 69.89 %. However, considering the clinical form of lesions and each PCR targets, positivity rates varied. Those results are in agreement with those of Turankar *and al.* [14] where in their study they compared several targets: RLEP, RNAr 16S, rpoT and Sod A for Hansen bacillus detection in clinical and environmental samples. They showed that, the RLEP was a gold choice target for detect *M. leprae* presence in clinical (Blood, cutaneous smears) and environmental samples (soil)

with a high positivity level. Always in this study *RLEP* target has been considered as best target in relation to other genes targets. However, *RLEP* target could detect *M. leprae* presence in 53 % of positive blood samples when BI was negative. On other hand, *Pra* target was to measure detect *M. leprae* presence in samples (BI negative) with a strong rate in relation to *RLEP* target.

Also identification of a repeated *RLEP* sequence which demonstrated a higher sensitivity and specificity than gene targets reported previously, allowed detection of low levels of *M. leprae* DNA. The molecular diagnosis (PCR) has been applied for the detection of *M. leprae* in different types of clinical samples, as the cutaneous smear [13], blood, them nasals [15], and saliva for the improvement of the diagnosis of the leprosy in laboratory. In this study, it has been described the use PCR for *M. leprae* detection in samples of ear dermal pulp and of them nasals among leprosy patients multibacillary and paucibacillary. Indeed, for the MB form, the *RLEP* PCR always shows 100 % positivity rates compared to 65.21 % for the *Pra* gene target with a statistically significant difference between PCR tests ( $P < 0.05$ ). For the PB forms, less rich in bacilli, the positivity rate observed for the *Pra* gene target was higher than that of the *RLEP* target respectively 74.80 % and 69.76 %. These results are similar to those of Banerjee *and al* [9], who affirmed that PCR-*Pra* was very useful in confirming the diagnosis of leprosy in patients, particularly for the TT form (75 % PCR-*Pra* positivity in both treated and untreated patients) while microscopic detection in slit skin smears was normally negative due to the low number of bacilli present in the clinical specimens [6]. Thus, the *Pra* PCR would be advantageous and could be used for the detection of paucibacillary cases as demonstrated by Callefi *and al.* [16] who worked on the detection of *M. leprae* in urine samples and have obtained the *Pra* gene with a detection rate of 75 %. These results corroborate those of Kamalanathan *and al.* [6] who showed that *Pra* PCR allows a better sensitivity with nasal mucus samples. The variation of PCR positivity of *M. leprae* observed has been related mainly to the different used primers, especially to the size of the fragments amplified and clinical samples [17]. Specificity and sensitivity of two targets (*RLEP* and *Pra*) in this study were verify using DNA coming from another mycobacteria, *M. ulcerans*. So It has been demonstrated specificity of primers used for *M. leprae* detection. *Pra* target detect *M. leprae* presence in samples (negative Bacillary Index) with a best detection rate in relation to *RLEP* target. *Pra* target could also detect *M. leprae* presence in the majority of nasal secretion samples, what indicates that *Pra* gene could be a better target for *M. leprae* detection in samples poor in bacilli. It could be due to the size of amplified sequences *Pra* target. In fact, the *Pra* target has a size of 151 bp smaller than that of the *RLEP* target. Small DNA fragments may be difficult to be damaged during the extraction procedure than larger and when DNA damaged arrives during the extraction step this makes it difficult to detect large targets by PCR [16]. Although several tools of diagnosis are available to detect cases of leprosy, they lack specificity and sensitivity [17]. PCR technology demonstrated a diagnostic precision improved for the epidemiological studies and required a minimum of time so it could be used for leprosy confirmation cases.

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## 5. Conclusion

This study reports the molecular detection of *M. leprae* using two genomic targets: repeated elements type *RLEP* and the *Pra* gene in the confirmation of clinical cases of leprosy in Côte d'Ivoire. The *RLEP* target has proven to be a prime target for the molecular detection of *M. leprae* because of the better positivity rate in PCR. However, paucibacillary form (poor bacilli samples) of leprosy or clinical cases of leprosy with a negative bacilloscopy index could be detected using the *Pra* gene as PCR target.

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## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declare that they do not have any conflict of interests.

### *Statement of ethical approval*

This study was approved by the National Ethic committee for research of Côte d'Ivoire under the approval number N/Réf: N°140/MSHP/CNER-km.

*Statement of informed consent*

Informed consent was obtained from all individual participants included in the study.

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